

Mutational Analysis of Essential Septins Reveals a Role for Septin-Mediated Signaling in Filamentation

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Septin proteins are conserved structural proteins that often demarcate regions of cell division. The essential nature of the septin ring, composed of several septin proteins, complicates investigation of the functions of the ring, although careful analysis in the model yeast *Saccharomyces cerevisiae* has elucidated the role that septins play in the cell cycle. Mutation analysis of nonessential septins in the pathogenic fungus *Candida albicans* has shown that septins also have vital roles in cell wall regulation (CWR), hyphal formation, and pathogenesis. While mutations in nonessential septins have been useful in establishing phenotypes, the septin defect is so slight that identifying causative associations between septins and downstream effectors has been difficult. In this work, we describe decreased abundance by mRNA perturbation (DAmP) alleles of essential septins, which display a septin defect more severe than the defect observed in deletions of nonessential septins. The septin DAmP alleles have allowed us to genetically separate the roles of septins in hyphal growth and CWR and to identify the cyclic AMP pathway as a pathway that likely acts in a parallel manner with septins in hyphal morphogenesis.

Candida albicans is a commensal fungus of the human gastrointestinal and genitourinary tracts. It causes serious systemic disease in patients with compromised immune systems, as well as in immunocompetent patients taking broad-spectrum antibiotics or with indwelling medical devices. Despite therapy, systemic infections can have mortality rates as high as 40% in some immunocompromised populations (1). The ability to switch between yeast and hyphal morphogenetic forms is vital for the pathogenesis of the organism (2, 3). Our focus here is a protein family, called septins, whose members have roles in both antifungal drug sensitivity and hyphal growth of *C. albicans*.

Septins are highly conserved structural components of animal and fungal cells that have roles in the polarization of cells and cell division. These GTP-binding proteins are stable proteins whose localization and activity is regulated by posttranslational modifications (4). Their essential role in the cell cycle is best understood in the model yeast *Saccharomyces cerevisiae*. In this fungus, septins localize to the presumptive bud site and remain in the bud neck between mother and daughter cells as the cell cycle progresses, serving as a scaffold to direct protein localization to sites of cell separation (5). Following the conclusion of the cell cycle, these essential proteins rapidly disperse from the bud neck and are recycled to the next presumptive bud (6). Septins appear to behave similarly in the yeast-like cells of the pathogenic fungus *C. albicans* and are also essential for cell survival (7). Septins, however, behave differently in the hyphal cells of *C. albicans*. In this growth form, septin rings remain stable for several cell generations, and the behavior of individual septins is altered (8). While these observations strongly support a role for septins during hyphal growth, we know very little about why their continued presence is important in hyphal cells of *C. albicans*.

Five septins have been characterized in *C. albicans* that correspond to the five mitotic septins in *S. cerevisiae*, and they are encoded by *CDC3*, *CDC10*, *CDC11*, *CDC12*, and *SEP7* (an ortholog of *S. cerevisiae* *SSH1*). These septins have conserved localization at

the bud neck in *C. albicans* yeast cells, and the septin ring is thought to play a conserved role in the cell cycle during yeast-like growth. *CDC3* and *CDC12* are essential in both *S. cerevisiae* and *C. albicans* and are thought to be critical components in stabilizing the septin ring. *CDC10* and *CDC11* are also important components of the septin ring. However, cells can survive without *CDC10* or *CDC11* under ideal growth conditions, a fact that has been utilized when searching for septin functions. In *C. albicans*, mutations in *CDC10* and *CDC11* have been used to uncover roles for septins in hyphal growth and cell wall integrity and/or biogenesis (collectively called cell wall regulation [CWR]). *C. albicans* cells lacking *CDC10* or *CDC11* are unable to form hyphae on solid surfaces, have defects in hyphal formation in liquid media, and are hypersensitive to the cell wall-targeting antifungal drug caspofungin (7, 9). A recent report has also shown that a temperature-sensitive mutation in *CDC12*, encoding an essential septin, caused severe hyphal defects in liquid media (10).

Septins are highly regulated proteins. Phosphorylation, sumoylation, acetylation, methylation, and GTP binding are all thought to play some role in regulating septin localization and the stability of the septin filament structures in eukaryotes (4). However, little is known about how septins are regulated or what signals may lie downstream of septins in *C. albicans*. The cyclin-dependent kinase Cdc28 and the Nim1 kinase Gin4 have demonstrated roles in regulating the septin ring during yeast-like and

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TABLE 1 Strains used in this study

Strain	Genotype	Source or reference
BWP17	<i>ura3/ura3 arg4/arg4 his1/his1</i>	13
CW412	<i>ura3/ura3 arg4/arg4 CDC12-HIS1/his1 CDC12-tUAU1/CDC12-tURA3</i>	This study
DAY185	<i>ura3/ura3::URA3 arg4/arg4::ARG4 his1/his1::HIS1</i>	23
JRB217	<i>ura3/ura3/URA3 arg4/arg4/UAU1 his1/his1 SEP7-GFP::HIS1/SEP7</i>	This study
JRB271	<i>ura3/ura3 arg4/arg4 his1/his1 cdc10::ARG4/cdc10::URA3</i>	This study
JRB363	<i>ura3/ura3 arg4/arg4 his1/his1 cdc10::ARG4/cdc10::URA3 SEP7/SEP7-GFP-HIS1</i>	This study
JRB412	<i>ura3/ura3 arg4/arg4 his1/HIS1 CDC3-tUAU1/CDC3-tURA3</i>	This study
JRB419	<i>ura3/ura3 arg4/arg4 HIS1/his1 CDC12-tUAU1/CDC12-tURA3</i>	This study
JRB421	<i>ura3/ura3 arg4/arg4 CDC3-HIS1/his1 CDC3-tUAU1/CDC3-tURA3</i>	This study
JRB461	<i>ura3/ura3 arg4/arg4 SEP7-GFP::HIS1-his1/his1 CDC3-tUAU1/CDC3-tURA3</i>	This study
JRB467	<i>ura3/ura3 arg4/arg4 SEP7-GFP::HIS1-his1/his1 CDC12-tUAU1/CDC12-tURA3</i>	This study
JRB470	<i>pTDH3-TPK1 ura3/ura3::URA3 arg4/arg4::ARG4 his1/his1::HIS1</i>	This study
JRB473	<i>pTDH3-TPK1 ura3/ura3 arg4/arg4 his1/HIS1 CDC3-tUAU1/CDC3-tURA3</i>	This study
JRB483	<i>pTDH3-TPK1 ura3/ura3 arg4/arg4 his1/HIS1 CDC12-tUAU1/CDC12-tURA3</i>	This study
JRB485	<i>pTDH3-EFG1 ura3/ura3::URA3 arg4/arg4::ARG4 his1/his1::HIS1</i>	This study
JRB490	<i>pTDH3-EFG1 ura3/ura3 arg4/arg4 his1/HIS1 CDC3-tUAU1/CDC3-tURA3</i>	This study

hyphal growth (11, 12), but it is quite likely that further elements act upstream of septins to regulate their localization and stability. No pathways working downstream of septins have yet been identified in *C. albicans*, although the Hsl1 and Swe1 protein kinases, which coordinate responses to septins in *S. cerevisiae*, are present in *C. albicans*. Identification of the pathways that operate upstream and downstream of septins will provide insight into the contribution of septins to CWR and hyphal morphogenesis.

We have found that a decrease in the level of the essential septins in *C. albicans* reduces accumulation of other septins at the neck. Reduced septin levels also lead to an increase in susceptibility to cell wall stress, defects in hyphal formation, and reduced virulence in a murine tail vein injection model. In addition, decreasing essential septins in the cell leads to gene expression changes that we used to identify a pathway that may act downstream of or in parallel with septins in hyphal morphogenesis. Overexpression of the protein kinase A (PKA) *TPK1* subunit restored hyphal growth in the septin mutant cells, but not cell wall stress resistance, suggesting that distinct pathways exist downstream of septins to mediate cell wall stress and hyphal signals.

MATERIALS AND METHODS

Strains and media. Strains were grown on yeast extract-peptone-dextrose (YPD) rich medium (10 g yeast extract [RPI], 20 g peptone [RPI], and 2% glucose [Sigma] in 1 liter of H₂O) and spider medium (10 g D-mannitol [Sigma], 10 g nutrient broth [BD Difco], 2 g K₂HPO₄ [Sigma] in 1 liter of H₂O). Caspofungin (Merck) and nourseothricin (Werner Bioagents) were added to media at the concentrations described. Primer sequences are listed in Table S1 in the supplemental material.

Mutant strains were made in the BWP17 background (13) (Table 1). Decreased abundance by mRNA perturbation (DAmP) mutant strains were made by replacing the 3' untranslated region (UTR) of the respective gene with a plasmid sequence (Fig. 1). Two primers approximately 120 bp in length were designed to amplify the plasmid sequence surrounding the *URA3* marker in the pGEM-URA plasmid (13). The 3'-most regions of the primers were designed to amplify the plasmid sequence, while the bulk of the primer sequence was specific to the 3' region of the gene of interest. Primers jrb599 and jrb600 were used to generate the initial *CDC3-DAmP* allele. Primers jrb602 and jrb603 were used to generate the initial *CDC12-DAmP* allele. These insertions were selected for on synthetic complete (SC)-Ura agar plates and checked by colony PCR. Primers jrb493 and jrb513 were used to detect the DAmP insertion for *CDC3*, and primers

jrb496 and jrb513 were used to detect the DAmP insertion for *CDC12*. We replaced the *URA3* gene in the DAmP allele with a *UAU1* cassette, which contains two overlapping fragments of the *URA3* gene surrounding an intact *ARG4* gene (Fig. 1B). This construct was used to render the DAmP alleles homozygous by low-frequency mitotic-recombination events.

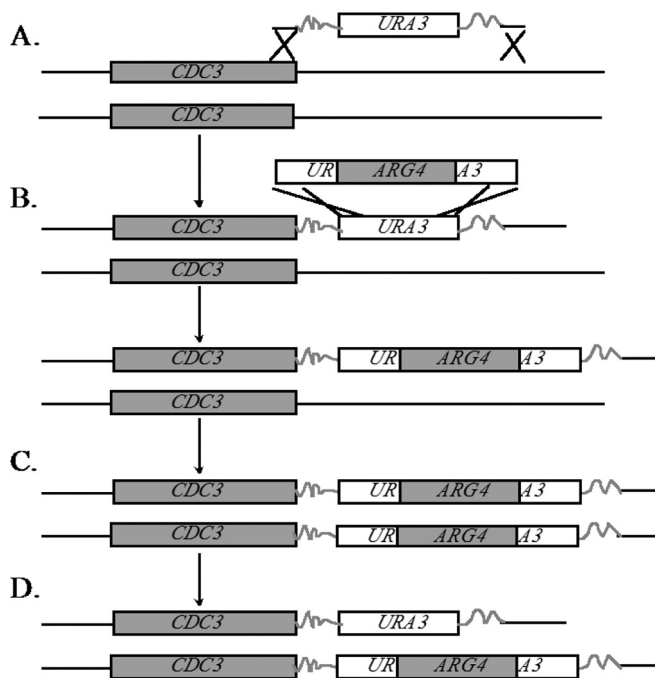


FIG 1 DAmP mutational strategy. (A) A *URA3* construct was amplified with primers containing a sequence homologous to the 3' end of *CDC3*. This construct was transformed into *C. albicans* strain BWP17, and positive transformants were selected on SC-Ura agar plates and confirmed by PCR. (B) The *URA3* marker was replaced with a *UAU1* cassette containing a fully functional *ARG4* marker flanked by overlapping portions of the *URA3* gene, yielding an *Arg⁺ Ura⁻* strain. (C) Via low-frequency mitotic recombination, homozygosity of the mutation can occur. Because the markers are the same, this event cannot be selected for. (D) Another mitotic-recombination event in one *CDC3* allele between the overlapping regions of *URA3* loops out the *ARG4* marker and yields a strain that can be selected for on SC-Arg-Ura medium. The resulting strains were confirmed by PCR.

Construction of the homozygous DAMP strains followed previously established methods (14). Briefly, putative Arg⁺ transformants were selected on SC–Arg-plus-uridine media and checked by colony PCR. Positive colonies were grown for 24 h in YPD liquid medium and patched onto SC–Arg–Ura plates. Putative homozygous DAMP strains were tested by colony PCR to ensure the absence of a wild-type (WT) band and the presence of transformed alleles. Primers jrb493/jrb601 (*CDC3*) and jrb496/jrb604 (*CDC12*) were used to detect the wild-type bands for the respective genes. The primer sequences are listed in Table S1 in the supplemental material.

To complement the mutant strains, a fragment of DNA 800 to 1,000 bp upstream and 200 to 350 bp downstream of the respective gene was amplified from BWP17 genomic DNA. Primers for *CDC3* complementation (jrb471/jrb472) were 60 to 70 bp in length, and the 3' 20 to 25 bp was gene specific. A 40-mer sequence was tacked onto the 5' end of each primer sequence to guide *in vivo* recombination into the pDDB78 plasmid (15). The amplified complementation fragment was cotransformed into the *S. cerevisiae* BY4741 Δ *trp1* strain (from the yeast deletion library [16]) with an EcoRI/NotI-linearized pDDB78 plasmid, and the resulting complementation clone was amplified in *Escherichia coli*. The primers for *CDC12* complementation (jrb666/jrb667) had restriction sites ClaI (jrb666) and NotI (jrb667) added to the 5' end. The *CDC12* sequence was amplified by PCR, digested with ClaI and NotI, and ligated to a ClaI/NotI-linearized pDDB78 plasmid. Each complement clone was digested with NruI to target insertion to the *HIS1* locus and transformed into the appropriate mutant strains, complementing the mutation and rendering the strains His⁺, as well. Complementation was checked by colony PCR using primers jrb493/jrb601 (*CDC3*) and jrb496/jrb604 (*CDC12*). pDDB78, digested with NruI, was transformed into the same mutant strains to generate prototrophic, marker-matched strains for comparison with the complemented strains.

To express a *SEP7-GFP*-tagged strain, plasmid pJRB103 (9) was digested with BclI (New England BioLabs), and the resulting linearized vector was transformed into *his1* auxotrophic strains as indicated in the strain table (Table 1). Transformants were selected on media lacking histidine, and putative insertion mutant strains were checked by colony PCR using primers jrb388/jrb391.

Overexpression of *TPK1* and *EFG1* was accomplished by replacing the native promoter of one allele of each gene with a *TDH3* promoter. A NAT-marked *TDH3* promoter construct was amplified from pCJN542 using ~120-mer oligonucleotides. Twenty base pairs of the 3' ends of these oligonucleotides was designed to amplify the construct, while the remaining 100 bp was gene specific, designed to guide *in vivo* recombination in *C. albicans*. Primers jrb656/jrb657 were used to direct the insertion of the *TDH3* promoter upstream of *TPK1*. Primers SF1501/SF1502 were used to direct the insertion of the *TDH3* promoter upstream of *EFG1*. These promoter replacement transformants were selected on YPD agar with 400 μ g/ml nourseothricin. Putative positive transformants were tested by colony PCR (*TPK1* primers jrb662/jrb19 and *EFG1* primers jrb670/jrb19) for the insertion of the overexpression construct upstream of the intended gene.

Sensitivity and hyphal-growth assays. For testing sensitivity to caspofungin, strains were grown overnight in liquid YPD medium at 30°C and then diluted to an optical density at 600 nm (OD₆₀₀) of 3 in H₂O. Five-fold dilutions of this stock were plated onto YPD or YPD plus 125 ng/ml caspofungin agar. The plates were incubated for 48 h at 30°C and digitally photographed.

For hyphal-growth assays, 20 μ l of the final (least dense) dilution from the sensitivity dilution stocks mentioned above was plated onto spider agar plates. The plates were incubated at 37°C for 4 to 5 days. Colony edges were imaged using a Nikon Eclipse TS100 with a 10 \times 0.25-numerical-aperture (NA) Ph1 objective. Representative images are shown.

Septin fluorescence microscopy. *SEP7-GFP* cells were grown overnight and diluted to an OD₆₀₀ of ~0.5 in YPD medium. Two hundred microliters of this culture was pipetted onto glass bottom dishes (MatTek

Corporation, Ashland, MA) coated with concanavalin A. The dishes were washed twice with phosphate-buffered saline (PBS) and resuspended with PBS for immediate visualization. The cells were visualized with a Zeiss Axio Observer Z.1 fluorescence microscope and either a 100 \times 1.4-NA or a 63 \times objective. Fluorescent images were acquired with an exposure time of 0.5 s on a Coolsnap HQ² (Photometrics) camera using AxioVision software (Zeiss) or on an AxioCam digital monochrome camera (Zeiss) using Zen pro software (Zeiss). Fluorescence measurements were calculated using ImageJ software (NIH). The average fluorescence intensities within the mother and daughter cells (roughly equivalent measurements) were compared to peak intensity values within the bud neck. Mother/daughter cell averages and bud neck values were subtracted from the average background intensity for each image. *P* values for fluorescence differences between *CDC3-DAmP*, *CDC12-DAmP*, and *cdc10 Δ / Δ* cells and the wild type were <1 \times 10⁻¹³.

RNA extraction. Overnight cultures of cells were diluted to an OD₆₀₀ of 0.2 in 50 ml fresh YPD medium. The cultures were allowed to grow at 30°C with shaking until the culture density reached an OD₆₀₀ of ~1. Cells were then harvested by vacuum filtration and flash frozen in a dry-ice-ethyl alcohol (EtOH) bath. The cells were kept frozen on filters at -80°C until RNA extraction. RNA was extracted using a Qiagen RNeasy kit (Qiagen) following the manufacturer's instructions with the following modifications. The cells were resuspended from filters with 1.5 ml ice-cold distilled H₂O (dH₂O), followed by 20 s of vigorous vortexing. The resuspended cells were transferred to a 1.5-ml tube and spun down following the manufacturer's protocol. Furthermore, during the cell disruption step, the cells were lysed with a bead beater for 3 min at 4°C to maximize cell lysis.

Gene expression analysis. The expression of genes in the *CDC3-DAmP* and *CDC12-DAmP* mutant strains were analyzed by NanoString and quantitative reverse transcription-PCR (qRT-PCR) analysis (described below). They were compared to the expression of protein kinase mutant strains exhibiting hypersensitivity to caspofungin (*cla4*, *hsl1*, *kin3*, *swe1*, *tpk1*, *yck2*, *yck3*, *prk1*, *ssn3*, and *hst7*) that were analyzed by microarray (described below).

NanoString. NanoString analysis is a sensitive method for analyzing gene expression of 100 to 800 targets at a time. For our analysis, 295 targets associated with the cell wall and with biofilm formation were selected for analysis. Approximately 100 ng of *C. albicans* total RNA was mixed with the NanoString probe set (NanoString Technologies, Seattle, WA) and incubated at 65°C overnight. The reaction mixture was then loaded on the NanoString nCounter Prep Station for binding and washing, using the default program. The resultant cartridge was then transferred to the NanoString nCounter digital analyzer for scanning and data collection. A total of 600 fields were captured per sample. The raw data were first adjusted for binding efficiency and background subtraction, following nCounter data analysis guidelines. Five control genes, *ACT1*, *TDH3*, *YRA1*, *ARP3*, and *orf19.7235*, were used to normalize gene expression.

qRT-PCR. Ten micrograms of total RNA was treated with a DNA-free kit (Ambion), followed by first-strand cDNA synthesis from half of the DNA-free RNA using iScript Reverse Transcription Supermix (Bio-Rad). Absence of DNA contamination was confirmed using control sets for which reverse transcriptase was omitted from the cDNA reaction.

Primer3 software (<http://frodo.wi.mit.edu/>) was used to design primers for *CDC3*, *CDC12*, *orf19.1862*, *orf19.251*, *orf19.7310*, and *HSP12*. Additional primer sets used in these studies were generated in previous studies. The primers are listed in Table S1 in the supplemental material. iQ SYBR green Supermix (2 \times ; Bio-Rad), 1 μ l of first-strand cDNA reaction mixture, and 0.1 μ M primers were mixed in a total volume of 50 μ l per reaction, and real-time PCR was performed in triplicate for each sample on an iCycler iQ real-time PCR detection system (Bio-Rad). The program for amplification had an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 45 s and 58°C for 30 s. Product amplification was detected using SYBR green fluorescence during the 58°C step, and the specificity of the reaction was monitored by melting curve analysis

following the real-time program. *TDH3* was used as a reference gene for normalization of gene expression, which was determined using Bio-Rad iQ5 software (the $\Delta\Delta C_T$ method).

Microarray analysis. Microarray analysis was performed on *C. albicans* Affymetrix NimbleExpress arrays (CAN04a530004N) as previously described (17). Strains containing mutations in protein kinases were compared to a wild-type marker-matched strain (DAY286). The strains were generated previously (9) and are identified in Table S4 in the supplemental material.

Virulence assays. Seven-week-old male ICR mice (Harlan-Sprague) were inoculated by intravenous injection in the lateral tail vein with 1×10^6 cells of *C. albicans* strains in 0.2 ml of normal saline. The mice were followed until they were moribund, at which point they were sacrificed. Survival curves were calculated according to the Kaplan-Meier method using the PRISM program (GraphPad Software) and compared using the log-rank (Mantel-Cox) test. A *P* value of <0.05 was considered significant. This murine study was approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed according to its guidelines.

RESULTS

DAmP alleles of essential septins cause reduced septin accumulation at the bud neck. Studies with mutants with mutations in the nonessential septin genes *CDC10* and *CDC11* in *C. albicans* have demonstrated that the septin ring is vital for CWR (9), hyphal morphogenesis (7), and pathogenesis (18). It has been assumed that the septin ring is weakened in *cdc10* Δ/Δ and *cdc11* Δ/Δ mutant cells and that deletions of these septins exhibit a mild version of a complete loss of the septin ring. While we believe that this may be true for certain functions, we believe that the essential septins have additional roles in CWR and morphogenesis that can be uncovered only by studying mutations in these septins.

Septin proteins are stable over multiple cell generations (6), with localization cues driven by posttranslational modifications. This stability renders traditional approaches to study *C. albicans* essential gene function, such as promoter substitutions, difficult to assess. Therefore, a DAmP strategy was used to mutate *CDC3* and *CDC12* in *C. albicans*. In this approach, the 3' UTRs of both alleles of *CDC3* and *CDC12* were replaced with foreign non-UTR sequence, removing the polyadenylation addition sites. Replacement of the 3' UTR with vector sequence should destabilize the RNA by preventing polyadenylation. Accordingly, we found that removal of this sequence leads to a decrease in RNA accumulation of the targeted septin (Fig. 2A). The RNA levels of *CDC3* were not altered in a *CDC12-DAmP* strain, and the RNA levels of *CDC12* were not altered in a *CDC3-DAmP* mutant strain (see Fig. S1A in the supplemental material), nor did the septin DAmP mutations significantly affect the RNA levels of other mitotic septins (see Fig. S1B in the supplemental material). This demonstrates that reduction of expression of a single septin does not affect the regulation of other septins and that the phenotypes we observe are due to the reduced expression of the targeted septin.

A *SEP7-GFP* fusion gene was introduced into the DAmP septin mutant strains in order to assess septin ring formation. We compared the new DAmP mutant strains to *SEP7-GFP*-containing *cdc10* Δ/Δ and WT strains. Localization of the septin ring in yeast cells was not affected by the deletion of *CDC10* or by the DAmP mutations in *CDC3* and *CDC12* (Fig. 2B). However, the fluorescence intensity of Sep7-GFP at the bud neck was reduced by approximately 50% in both the *CDC3-DAmP* and the *CDC12-DAmP* strains compared to the wild type, suggesting that the septin ring was physically affected by reduced levels of essential

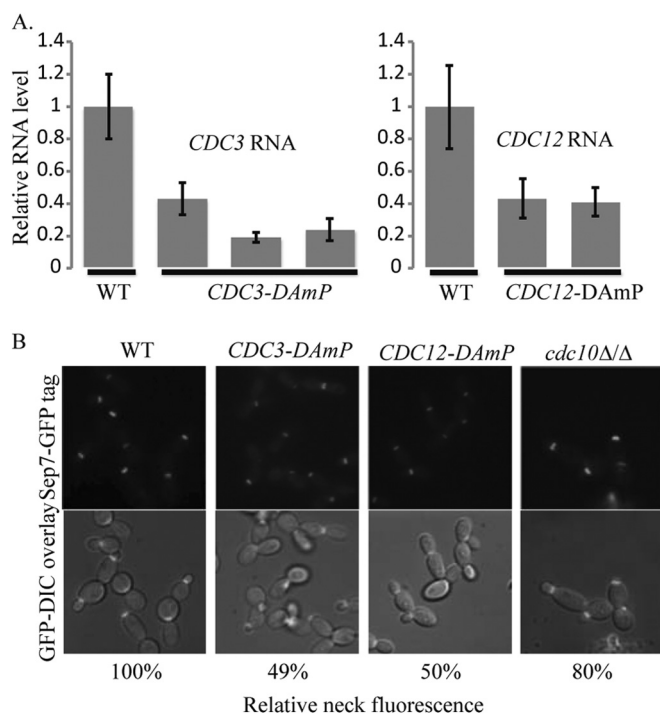


FIG 2 Septin DAmP alleles reduce septin gene expression and septin accumulation at the bud neck. (A) The expression of *CDC3* or *CDC12* in yeast cells was tested in three independently isolated *CDC3-DAmP* strains (JRB412, JRB413, and JRB415, from left to right in the left graph) and two independently isolated *CDC12-DAmP* mutant strains (JRB418 and JRB419, from left to right in the right graph). Expression in the mutant strains was normalized to expression in a marker-matched wild-type control, DAY185 (WT). (B) The localization and fluorescence intensity of Sep7-GFP were measured in *CDC3-DAmP* (JRB461; 159 cells), *CDC12-DAmP* (JRB467; 131 cells), and *cdc10* Δ/Δ (JRB363; 152 cells) mutant yeast cells. Fluorescence levels from a Sep7-GFP-tagged marker-matched wild-type strain (JRB217; 78 cells) (WT) were used to normalize mutant values. *P* values between the marker-matched control and the mutant cells were highly significantly different ($P < 1 \times 10^{-17}$ for *CDC3-DAmP*, $P < 1 \times 10^{-16}$ for *CDC12-DAmP*, and $P < 1 \times 10^{-14}$ for the *cdc10* Δ/Δ strain), as were the values between the *CDC3-DAmP/CDC12-DAmP* and the *cdc10* Δ/Δ mutant cells ($P < 1 \times 10^{-61}$ and $P < 1 \times 10^{-36}$, respectively).

septins (Fig. 2B; see Table S2 in the supplemental material). This reduction of fluorescence was not as severe in the *cdc10* Δ/Δ strain (~37% reduction in fluorescence), suggesting that the DAmP alleles of the essential septin genes cause a more severe defect and might allow us to uncover novel septin functions.

DAmP septins have hyphal-growth and CWR defects. Septins are important for both hyphal growth and CWR, although their specific roles in these processes are poorly defined. Based on the reduction of septin accumulation at the neck observed in the septin DAmP mutant strains, we anticipated that these strains would also be defective in hyphal growth and CWR. To test for CWR defects in the septin DAmP mutant strains, we utilized a drug, caspofungin, that targets the enzyme responsible for producing the β -1,3-glucan, a major component of the cell wall. This drug has devastating effects on the cell wall of the fungus and is currently used as a clinical antifungal drug. Mutant and complemented strains were tested for the ability to grow in the presence of 125 ng/ml caspofungin. Compared to the wild-type and complemented strains, the DAmP mutant strains displayed hypersensitivity to caspofungin, indicating that the reduction in septins at the bud neck does indeed have an impact on CWR (Fig. 3A).

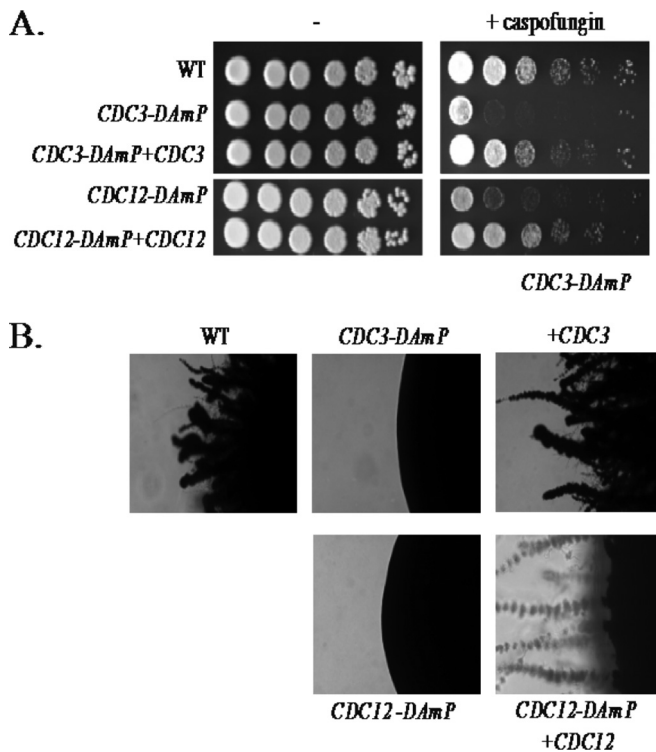


FIG 3 Septin DAmP alleles are hypersensitive to cell wall stress and defective for hyphal development on solid surfaces. (A) Growth of a marker-matched wild-type strain (DAY185) was compared to that of *CDC3-DAmP* (JRB412), *CDC3-DAmP+CDC3* (JRB421), *CDC12-DAmP* (JRB419), and *CDC12-DAmP+CDC12* (CW412) mutant strains in the presence (+ caspofungin) and absence (–) of 125 ng/ml caspofungin. The strains were allowed to grow for 2 days at 30°C and imaged. The spots represent 5-fold dilutions from left to right. (B) The strains indicated for panel A were spotted onto spider agar plates and incubated at 37°C for 6 days. Images of the edges of representative single colonies are shown.

There are a number of signals that induce *C. albicans* to switch from yeast-like growth to hyphal growth. High temperatures, low pH, nutrient starvation, contact with a surface, and the presence of serum can all induce hyphal growth. Mutants with deletions of nonessential septins display a modest hyphal defect under liquid induction conditions but are very defective in hyphal formation on a solid surface (7). DAmP mutants of essential septins also displayed a moderate hyphal defect in liquid media (data not shown) and were very defective in hyphal formation on a solid surface (Fig. 3B; see Fig. S2 in the supplemental material). Complementation restored normal hyphal growth to the septin DAmP mutants.

Transcriptional profiles of DAmP septins reveal a connection with the cyclic AMP (cAMP) pathway. Transcriptional profiles of 300 genes in *CDC3-DAmP*, *CDC12-DAmP*, and *cdc10Δ/Δ* strains were measured with NanoString technology in the absence of stress. The 300 genes tested were selected largely for putative roles in cell wall biogenesis or for their presence in the cell wall, along with several control genes. Profiles were performed in triplicate for each mutation with independent mutant strains when available. While there was significant overlap between *CDC3-DAmP* and *CDC12-DAmP* homozygote mutant profiles, the *cdc10Δ/Δ* mutation had little to no impact on gene expression (see Table S3 in the supplemental material), consistent with prelimi-

nary microarray and qRT-PCR analyses of the strain (data not shown). Complementation of the *CDC3-DAmP* mutation with a wild-type copy of *CDC3* at the *HIS1* locus restored wild-type levels of gene expression to the subset of interesting genes from the NanoString data set that we tested by RT-PCR (Table 2).

The transcriptional profile of the *CDC3-DAmP* and *CDC12-DAmP* homozygote mutant strains was compared to preliminary microarray data for 11 protein kinase mutant strains (see Table S4 in the supplemental material). The protein kinase mutations were homozygous insertion mutations, and these mutant strains, like the septin mutant strains, were also hypersensitive to caspofungin (9). Eleven mutant strains were tested, i.e., those with mutations in *cla4*, *hsl1*, *ire1*, *kin3*, *swe1*, *tpk1*, *yck2*, *yck3*, *prk1*, *ssn3*, and *hst7*. Of the mutant strains tested, only the *tpk1^{-/-}* strain showed a sizeable overlap with the septin DAmP mutants. Like the *CDC3-DAmP* and *CDC12-DAmP* mutations, few genes were impacted by the *tpk1^{-/-}* mutation, but the few genes that were affected generally overlapped. In all, approximately 58% of the genes upregulated in the *tpk1^{-/-}* mutant were also upregulated in the *CDC3-DAmP* and *CDC12-DAmP* mutant strains (Table 3), suggesting some overlap in function between septins and the cAMP pathway.

Overexpression of *TPK1* and *EFG1* restores hyphal growth and virulence to septin DAmP strains. Tpk1 is one of two protein kinase A subunits in the cyclic AMP pathway, a conserved pathway in eukaryotes that generally serves as a signal transduction pathway sensing nutrient conditions. Like septins, Tpk1 has a role in filamentation on solid surfaces and in CWR, while Tpk2 is involved in filamentation in liquid media (9, 19, 20). Our hypothesis, based on our transcriptional-profiling data, was that the cAMP pathway either acts upstream of septins or lies downstream of septin-mediated signaling. We had previously shown that septin localization was unaffected by the *tpk1^{-/-}* mutation (9), and thus, the latter hypothesis seemed most likely. If true, overexpression of components of the cAMP pathway should restore wild-type hyphal growth and CWR to septin DAmP strains. Therefore, we replaced the promoter of one allele of the protein kinase gene *TPK1* or the transcription factor gene *EFG1*, a downstream target of Tpk1, with the promoter for the highly expressed gene *TDH3* in the *CDC3-DAmP*, *CDC12-DAmP*, and wild-type strains. These strains were then tested for sensitivity to caspofungin and for the ability to form hyphae on spider agar at 37°C. As hypothesized, overexpression of *EFG1* or *TPK1* restored hyphal formation to the

TABLE 2 Complementation of a DAmP mutant restores gene expression

ORF ^a	Gene	Fold change ^b		P value ^c
		<i>CDC3-DAmP</i>	<i>CDC3-DAmP</i> + <i>CDC3</i>	
orf19.1055	<i>CDC3</i>	0.32	1.18	0.0004
orf19.5741	<i>ALS1</i>	8.44	1.56	0.0005
orf19.4255	<i>ECM331</i>	2.94	0.71	0.0006
orf19.1862		4.5	1.01	0.0387
orf19.7310		4.23	1.08	0.0044
orf19.3160	<i>HSP12</i>	4.66	0.94	0.0477
orf19.2060	<i>SOD5</i>	0.43	1.66	0.0404

^a ORF, open reading frame.

^b The data represent the fold change normalized to a marker-matched wild-type control strain (DAY185). Upregulated genes are shaded.

^c P values were measured by *t* test comparisons of mutant and complemented strains.

TABLE 3 Comparison of gene expression levels between *CDC3-DAmP*, *CDC12-DAmP*, and *tpk1^{-/-}* strains

Gene	ORF	Gene expression ^a		
		<i>CDC3-DAmP</i>	<i>CDC12-DAmP</i>	<i>tpk1</i> /WT
HSP12	orf19.3160	5.88	5.96	5.89
ORF19.4216	orf19.4216	2.77	3.83	4.37
AQY1	orf19.2849	4.34	3.80	2.81
orf19.1862	orf19.1862	5.36	3.39	3.36
ASR2	orf19.7284	5.13	2.90	2.61
HGT6	orf19.2020	2.38	2.84	1.29
ECM331	orf19.4255	2.48	2.63	2.97
GLK1	orf19.734	2.51	2.43	1.94
OSM1	orf19.4555	2.22	2.39	5.68
ALS4	orf19.4555	2.18	2.36	5.68
orf19.2125	orf19.2125	2.99	2.16	1.04
ALS1	orf19.5741	3.16	2.16	7.32
RHR2	orf19.5437	2.05	1.96	1.59
CHK1	orf19.896	2.32	1.73	1.54
ORF19.7310	orf19.7310	7.14	1.18	4.75
ORF19.251	orf19.251	2.62	0.71	4.46
PGA31	orf19.5302	0.86	0.50	2.07
orf19.4921.2	orf19.4921.2	0.84	0.35	0.44
PGA26	orf19.2475	0.28	0.24	0.89

^a Gene expression is fold change and normalized to a marker-matched wild-type control (DAY185). Upregulated genes are shaded.

septin DAmP strains (Fig. 4A and data not shown). However, overexpression of *EFG1* or *TPK1* did not restore wild-type caspofungin sensitivity to these strains (data not shown). This result is the first indication we have had that the roles of septins in hyphal formation and CWR can be genetically separated.

Hyphal development is a key component of *C. albicans* virulence, and deletion of nonessential septins causes attenuated virulence in the fungus, likely due to the defective hyphal formation in these strains (18). Like the mutations in nonessential septins, a DAmP mutation in the essential *CDC3* septin gene led to attenuated virulence in a murine systemic-infection model (Fig. 4B). Complementation with a functional copy of *CDC3* restored wild-type virulence to *CDC3-DAmP* mutant cells. Additionally, overexpression of *TPK1* also restores wild-type virulence in *CDC3-DAmP* mutant cells, strongly suggesting that the virulence defect of septin mutant strains is mediated through targets held in common with the cAMP pathway.

DISCUSSION

Septins have long been considered a structural element of the cell and indeed have been called “the fourth component of the cytoskeleton” (21). Our previous work and work by others suggest that septins are more than simple scaffolding structures for cell separation. Septins are dispersed from the bud neck when the cell wall is stressed, and they appear to pull functional cell wall biosynthesis machinery with them when they move (9, 22). This response may be part of the normal cellular defense against cell wall stress, and at least portions of this response are conserved in *S. cerevisiae*. The function of septins in *C. albicans* hyphae appears to be somewhat distinct from their roles in yeast cell division. Septins persist at sites of previous division in hyphae well after a septum has been laid between the filament segments, and their role at this stage is completely unknown (8). We sought to further elucidate the roles

of septins in cell wall stress and hyphal development by analyzing the roles of essential septins in these processes.

The septin ring is an essential structure in dividing cells, and the essential nature of the ring has made genetic analysis of septin function difficult. Analysis of nonessential components of the septin ring, encoded by *CDC10*, *CDC11*, and *SEP7*, have been informative in elucidating phenotypes associated with septin ring defects, including hyphal defects, virulence defects, and CWR defects. Expression analysis of these mutant strains, however, demonstrates no evidence of defects affecting gene expression, nor does the septin ring itself appear to be strongly affected by their loss in *C. albicans* (Fig. 2B). We used a different approach, decreased abundance by mRNA perturbation, to analyze the effect that mutating an essential septin might have on the cellular phenotype and gene expression.

The homozygous *CDC3-DAmP* and *CDC12-DAmP* strains shared some phenotypes with deletion mutants of nonessential septins, suggesting that the DAmP approach is a valid method for analysis of essential gene function in *C. albicans*. Both septin DAmP mutant strains had hyphal-development, CWR, and virulence defects, similar to strains with mutations in nonessential

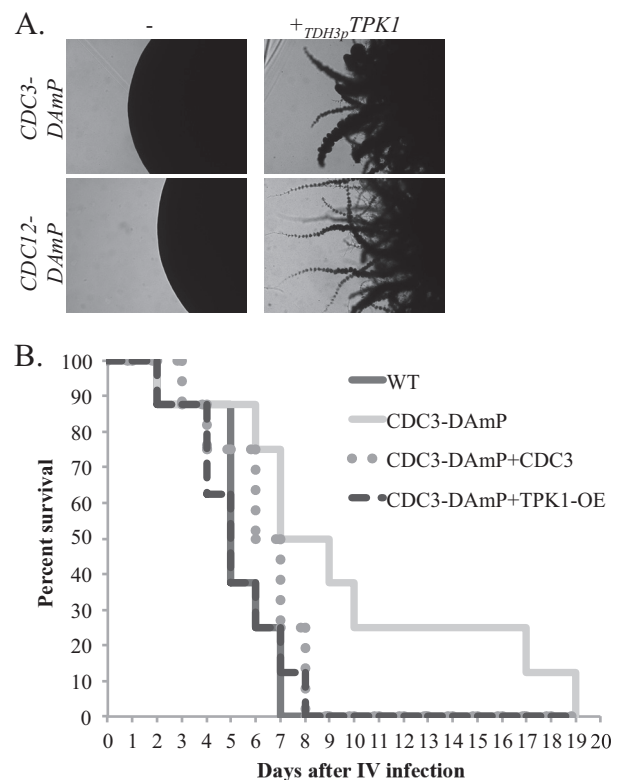


FIG 4 Overexpression of *TPK1* and *EFG1* restores hyphal growth and virulence defects of a *CDC3-DAmP* mutant strain. (A) *CDC3-DAmP* (JRB412), *CDC12-DAmP* (JRB418), and septin DAmP strains overexpressing *TPK1* were spotted onto spider agar plates and incubated at 37°C for 6 days. Images of the edges of representative single colonies are shown. (B) A marker-matched wild-type strain (DAY185) and *CDC3-DAmP* (JRB412), *CDC3-DAmP+CDC3* (JRB421), and *CDC3-DAmP+TPK1-OE* (JRB473) strains were injected into the tail veins of 8 mice each. Survival of infected strains was recorded daily. The time to death was significantly delayed for mice infected with the *CDC3-DAmP* strain ($P = 0.017$) but was not significantly different in the mice infected with the wild-type, *CDC3-DAmP+CDC3* ($P = 0.18$), or *CDC3-DAmP+TPK1-OE* ($P = 1.0$) strain.

septins. Unlike the nonessential-septin mutants, however, the DAmP mutant strains demonstrated a stark decrease of septin accumulation at the septin ring, as well as a transcriptional response to the mutation. These results are consistent with the rationale that impairment of essential septins might reveal new biological functions not evident from the nonessential-septin mutants.

We used transcriptional analysis to identify pathways that might act in parallel with septins for their roles in CWR or hyphal formation. The transcriptional profiles of *CDC3-DAmP* and *CDC12-DAmP* mutant strains were very similar to each other yet distinct from those of other DAmP mutant strains (data not shown), suggesting that this profile is septin specific and not caused by our mutational approach. Furthermore, the profile did not overlap the *cdc10Δ/Δ* mutant profile, suggesting the DAmP mutations were more severe than a deletion of a nonessential septin (see Table S3 in the supplemental material). Further evidence that this expression profile is septin specific is the observation that complementation of the DAmP strains with a wild-type copy of the mutated septin restores the wild-type expression profile. Using expression data from strains with similar phenotypes, we identified an overlap between the septin DAmP profile and the *tpk1^{-/-}* profile.

tpk1 encodes one of two PKA proteins in *C. albicans* that are an integral part of the cAMP signaling pathway. We hypothesized that septins were either being regulated in part by the cAMP pathway or that septins were acting upstream of some component in the cAMP pathway. Our evidence suggests that septin localization is not regulated by the cAMP pathway but that a pathway acting downstream of septins likely acts in parallel with the cAMP pathway. One possibility for this pathway is the Hsl1/Swe1 pathway, which acts downstream of septins to regulate the cell cycle in response to the state of septins at the bud neck. It is the only pathway that has thus far been shown to act downstream of septins. However, neither Hsl1 nor Swe1 mutant strains are defective in hyphal formation (22), suggesting that another, yet to be characterized pathway is acting downstream of septins to trigger hyphal growth in liquid media.

The transcriptional-profile similarities between *tpk1^{-/-}* and septin DAmP mutant strains suggests that a genetic relationship exists between septins and the cAMP pathway. We tested two hypotheses, that septin stability at the neck is affected by the cAMP pathway and that a septin signal feeds into the cAMP pathway upstream of cAMP, neither of which was supported by the results of our experiments. Septin stability at the bud neck in septin DAmP strains was unaffected by the overexpression of *TPK1*, and cAMP levels in yeast cells were at wild-type levels in a septin DAmP strain (data not shown). These data suggest that the cAMP pathway and a pathway functioning downstream of septins in hyphal formation act in parallel to regulate hyphal development and virulence in *C. albicans*.

Our work suggests that distinct pathways act in response to septins during cell wall damage and hyphal growth. Overexpression of *TPK1* and *EFG1* was sufficient to complement the hyphal-formation defect in septin DAmP mutant strains but did not restore wild-type sensitivity to cell wall damage. This suggests that the cellular responses downstream of septins in these two processes are distinct. Whether these pathways respond to septin changes or are activated by differentially regulated septins themselves remains to be seen. It is clear that the tools we have generated will provide us with the opportunity to identify novel pathways acting downstream of septins in distinct settings.

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